1 # Transplantation of Spermatogonial Stem Cells in Stallions

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## Abstract

Spermatogonial stem cells originate from gonocytes and undergo self-renewal and differentiation to generate mature spermatozoa via spermatogenesis in the seminiferous tubules of the testis in male mammals. Owing to the unique capacity of these cells, the spermatogonial stem cell transplantation technique, which enables the restoration of male fertility by transfer of germlines between donor and recipient males, has been developed. Thus, spermatogonial stem cell transplantation can be used as an important next-generation reproductive and breeding tool in livestock production. However, in large animals, this approach is associated with many technical limitations and inefficiency. Furthermore, research regrading spermatogonial stem cell transplantation in stallions is limited. Therefore, this review article describes the history and current knowledge regarding spermatogonial stem cell transplantation in animals and challenges in establishing an experimental protocol for successful spermatogonial stem cell transplantation in stallions, which have been presented under the following heads: spermatogonial stem cell isolation, recipient preparation, and spermatogonial stem cell transplantation. Additionally, we suggest that further investigation based on previous unequivocal evidence regarding donor-derived spermatogenesis in large animals must be conducted. A detailed and better understanding of the physical and physiological aspects is required to discuss the current status of this technique field and develop future directions for the establishment of spermatogonial stem cell transplantation in stallions.



**Keywords: Spermatogonial stem cells, Transplantation, Germ cells, Rete testis, Stallions**


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## Introduction

Spermatogenesis is a highly complex process that progressively produces and maintains functional sperm in the seminiferous tubules of the testis throughout the life of male mammals [1]. Spermatogenesis is initiated by the proliferation and differentiation of spermatogonial stem cells (SSCs). They are located adjacent to the basement membrane of the seminiferous tubules of the testis and undergo self-renewal and differentiation to maintain stable populations of spermatogenic cells for the production of mature spermatozoa [2, 3]. This unique capacity of SSCs has led to the development of transplantation techniques for use in animal reproduction and regenerative medicine. In contrast to the normal process of spermatogenesis, transplanted donor SSCs migrate from the lumen to the basement membrane of the seminiferous tubules. Before this process, endogenous donor SSCs are isolated from the testicular tissue and transplanted into the recipient's testis; they pass through the vas deferens, rete testis, and seminiferous tubules, and donor-derived spermatogenesis is re-established in the specific microenvironment, namely niche [4, 5].

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In 1994, the transplantation technique was first used to produce donor-derived offspring from infertile recipient mice [6]. This report demonstrated that donor-derived spermatogenesis could produce offspring through natural mating and opened up new possibilities for the use of SSCs in biomedicine and agriculture, such as for the production of genetically modified animals, conservation of superior genetic resources, and treatment of male infertility due to injury and disease. The transplantation technique has been extensively studied in various animals, including pigs [7-9], cattle [10-12], goats [13-15], shrews [16], monkeys [17-19], dogs [20, 21], and camels [22]. However, because of species-specific differences, such as anatomical differences, resistance of the lamina propria, and high volume-to-surface ratio, this technique has not been successful in all animals, and only a limited number of studies have demonstrated the production of donor-derived embryos and offspring [23]. A previous study showed that the transplantation technique could not be used in stallions because the rete testis could not be visualized via sonography [24]. To address these issues, we recently conducted a study in which donor germ cells were transplanted directly into the testicular tissue of the recipient's testes to induce donor-derived spermatogenesis in stallions [25]. However, our modified technique, which involved infusion of donor germ cells into the testicular tissue without using ultrasound guidance, was ineffective in stallions. This finding highlights the importance of using an ultrasound-guided technique and identifying optimal sites for germ cell transplantation in stallions to ensure successful outcomes.

The findings from the stallion study indicated that although the SSC transplantation (SSCT) is promising potential

for male fertility restoration in several species, the validation of experimental injection techniques and its effectiveness remains unclear and challenging in the satllions. Thus, detailed procedures and technical improvements need to be developed to achieve higher success rates of SSCT in the stallions.

In this review, we provide a comprehensive overview of the current advancement in the field of SSC transplantation (SSCT) research using several species including large animal models. In particular, it aims to discuss and summarize the SSC biology, ongoing attempts, main limitations, and highlight the requiremnets for the future experimental advancements to improve the SSCT efficiency in the stallions.

**Spermatogenesis in stallions**

Spermatogenesis is a stem cell-based process in which functional mature spermatozoa are progressively produced by division and differentiation within the seminiferous tubules that compose the testicular tissue. This process is accomplished through three distinct developmental stages, namely spermatocytogenesis, meiosis, and spermiogenesis [26, 27]. In stallions, spermatogenesis is typically completed in 57.4 days [28]. The first stage, i.e., spermatocytogenesis, which takes 19.4 days, is initiated by the development of spermatogonia. SSCs are the undifferentiated, immature germ cells adjacent to the basement membrane of the seminiferous tubules and are capable of self-renewal and differentiation for continuous production of mature sperm throughout the adult life [26]. A previous study showed that $A_1$, $A_2$, $A_3$, $A_4$, and B are the differentiating spermatogonial populations in mice and pigs [29], while in cattle and sheep, $A_1$, $A_2$, $A_3$, $B_1$, and $B_2$ were reported to be the differentiating spermatogonial populations [29]. Recently, eight different subtypes of spermatogonia, namely $A_s$, $A_{pr}$, $A_{al}$, $A_1$, $A_2$, $A_3$, $B_1$, and $B_2$, have been reported, and $A_s$, $A_{pr}$, $A_{al}$ spermatogonia are considered SSCs [30]. The two major functions of spermatogonia are duplication by mitosis ($A_0$) to maintain a progenitor population for the spermatogenic lineage and production of daughter cells by mitosis to generate primary spermatocytes that differentiate into spermatozoa [31]. In stallions, the $A_1$ spermatogonia are destined to produce primary spermatocytes. Once they are committed to this developmental line, they divide by mitosis in five stages to form 1 ($A_1$), 2 ($A_{1.2}$), 4 ($A_{1.4}$), 8 ($A_{1.8}$), and eventually 16 potential $A_2$ spermatogonia. The spermatogonia are connected by intercellular bridges to maintain their developmental lineage. Subsequently, the $A_2$ spermatogonia divide by mitosis, first into two $A_3$ spermatogonia, then into two $B_1$ spermatogonia, and finally into two $B_2$ differentiated spermatogonia [32]. Eventually, the $B_2$ spermatogonia divide into two primary spermatocytes that undergo the

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105 first meiotic division, which constititutes the second stage of spermatogenesis. In this stage, which also takes 19.4
106 days for completion, the secondary spermatocyte is formed by division of the primary spermatocyte into two
107 diploid secondary spermatocytes by mitotic division and exchange of genetic material. Two haploid spermatids
108 are then produced from each secondary spermatocyte; this process takes 0.7 days. Spermiogenesis is the final
109 stage of spermatogenesis and is completed in 18.6 days [33]. During this process, spermatids undergo dramatic
110 changes; their nuclei transform, they lose their cytoplasm, and develop tails, thus getting ready to differentiate
111 into mature spermatozoa [34].

112

113 **Preparation of donor SSCs for transplantation in stallions**

114 The technique of germ cell isolation from testicular tissue is widely used to collect pure individual germ cells.
115 The two-enzyme digestion method is usually applied for isolation in several species, including mouse [35], goat
116 [36], cow [37], buffalo [38], rhesus macaque [39], and pig [40]. This helped in the establishment of a germ cell
117 culture system and determination of various factors affecting spermatogenesis [41]. In addition to germ cell
118 evaluation, it is used to prepare donor germ cells for transplantation into the testis of recipient animals, such as
119 goat [13], boar [42], dog [20], and sheep [43]. The collection of pure germ cells, including SSCs, from the
120 testicular tissue using the two-enzyme digestion technique is essential for successful transplantation because stem
121 cells in donor germ cells are the only cell type that can completely regenerate spermatogenesis in the seminiferous
122 tubules of the recipient testis after transplantation [44]. However, like other adult stem cells, SSCs are present in
123 very small numbers in the testes, and as the testes mature, the number of SSCs decreases. For example, there are
124 only approximately 35,000 SSCs per testis in adult mice, and the percentage of the total number of germ cells in
125 the testis is 0.03% [45]. In neonatal pigs, approximately 7% of the gonocytes were present only in the seminiferous
126 tubules [46]. Thus obtaining large numbers of progenitor cells and other undifferentiated spermatogonia using the
127 isolation technique is difficult [47]. Recently, we evaluated the efficiency of the two-enzyme digestion technique
128 for isolation of the testicular tissue in stallions and demonstrated its effectiveness in isolating high-purity germ
129 cells from the testes [48]. Additionally, specific molecular markers for SSCs, including other germ cells, in the
130 testes of stallions have been developed. Antibodies, such as UTF-1 [49], DAZL [50], PGP9.5 [51], C-kit [52],
131 ACRBP [53], VASA [54], and Lin28 [55], can be used to identify specific germ cells at each developmental stage
132 of spermatogenesis in vitro. In particular, UTF-1 is a specific putative marker for undifferentiated SSCs in stallions
133 [49], and we found that 6.43% of germs cells per 10 g of testicular tissue in prepubertal stallions were positive for

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134 UTF-1 [48]. The immature testes are typically used to isolate SSCs for transplantation because they contain a
135 greater number of gonocytes or undifferentiated SSCs than do the mature testes. Nevertheless, this method does
136 not significantly increase the number of isolated SSCs. Hence developing other strategies to enrich the
137 concentration of SSCs to improve the efficiency of germ cell transplantation in stallions is necessary. As an
138 alternative to the use of immature testes, long-term in vitro culture of SSCs is widely known to elevate the number
139 of stem cells [56, 57]. Several key growth factors, such as GDNF, GFRa1, bFGF, CSF1, LIF, EGF, and IGF1,
140 which contribute to self-renewal and proliferation of SSCs in vitro have been identified [58, 59]. However, the
141 nature of the in vitro culture environment and efficacy of these growth factors on the expansion of SSC numbers
142 in stallions remains to be elucidated. Thus, the next challenge would be to establish an in vitro culture environment
143 to proliferate or differentiate SSCs for transplantation into stallions.

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145 **Preparation of recipient for transplantation of donor germ cells in stallions**

146 Preparation of the ideal recipient is a key issue in improving the success rate of SSCT. The fundamental purpose
147 of this step is to suppress all the endogenous germ cells while maintaining the full structure and function of the
148 somatic cells in the seminiferous tubules of the recipient's testes [60]. This provides the space for development of
149 stem cell niches, in which donor-derived spermatogenesis can be re-established in the basement membrane of the
150 seminiferous tubules in the recipient's testes after transplantation. Thus, several techniques to deplete endogenous
151 germ cells, such as local glycerol injections [61, 62], local irradiation [63, 64], heat treatment [65, 66], systemic
152 busulfan injections [6, 14, 67], and the use of genetically defective animals with congenital deletion of endogenous
153 spermatogenesis, have been developed [66]. However, no studies have demonstrated the efficacy of these
154 techniques in depleting endogenous germ cells in stallions. In our previous study, we found that intra-testicular
155 injection of 70% glycerol suppressed some germ cells in the seminiferous tubules in stallions. However, it did not
156 completely deplete endogenous germ cells for transplantation [68]. As an alternative, the chemotherapeutic agent,
157 namely busulfan is widely used to deplete endogenous germ cells to prepare recipient mouse models for
158 transplantation. However, the efficacy of this treatment varies in large animals; moreover, knowledge regarding it
159 is limited owing to the differences in physiological structure, composition, and size between species [18].
160 Additionally, busulfan is systemically toxic; it causes bone marrow suppression and inhibits hematopoiesis, which
161 occasionally leads to lethal effects. Therefore, the species-specific sublethal dose of busulfan required to deplete
162 endogenous germ cells should be determined. A recent study showed that multiple injections of low concentrations

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163 of busulfan can effectively deplete endogenous germ cells without any side effects in stallions [67]. However,
164 some problems with transplantation persist. Although treatment with busulfan is more effective than that with
165 glycerol, it cannot completely deplete germ cells, and recovery of the endogenous germ cells is inevitable. This
166 eventually disrupts the re-establishment of transplanted donor SSCs. Therefore, along with depletion of
167 endogenous germ cells of the recipient, determining the optimal time period for regeneration of donor-derived
168 spermatogenesis is important for successful transplantation in stallions.

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170 **Donor germ cell transplantation into recipient stallion testes**

171 Three methods are typically employed to ensure sufficient engraftment of donor SSCs into the lumen of
172 seminiferous tubules of the recipient's testes. In mice, donor SSCs have been successfully transferred into the
173 seminiferous tubules of the recipient's testes via efferent duct injection [6]. The efferent ducts are directly
174 connected to the rete tubules and epididymis of the testes. The seminiferous tubules converge to form the rete
175 tubules, which carry mature sperm from the seminiferous tubules to the efferent ducts [69]. Thus, efferent duct
176 injection is effective in transferring donor SSCs into the seminiferous tubules of the recipient's testes. However,
177 this transplantation technique, which is commonly used in rodents, cannot be applied to all animals because of
178 differences in anatomical structures, greater resistance of the lamina propria, and large volume-to-surface ratio
179 [18]. In mammals, the highly coiled, tightly packed seminiferous tubules are lined with individual compartments
180 consisting of inwardly extending tunica albuginea [47]. Therefore, the potential rise in intratubular pressure during
181 transplantation may rupture the seminiferous tubules and membrane of the testes [69]. In bovine animals, donor
182 SSCs are directly injected into the seminiferous tubules of the recipient's testes; however, this method was found
183 to be difficult and inefficient because of the highly convoluted tubular structure of the bovine testes. The rete testis
184 has a distinct structure and color and can be easily distinguished from the surrounding tissues in the testicles, thus
185 allowing more practical access to the seminiferous tubules [70]. Hence a large volume of germ cell suspension
186 can be injected into the rete testis, thereby improving the efficiency of cell transfer and success rate of
187 transplantation. However, the structural location of the rete testis varies between species. Unlike the rete testis of
188 rodents, such as mice, rats, and hamsters, which is located closer to the subcapsular area of the testis, that of most
189 other species, such as cats, dogs, bulls, boars, and monkeys, is located deep in the center of the testis [4, 69, 71].
190 Hence, transplantation of SSCs through the rete testis is more difficult and less feasible in large animals than it is
191 in rodent models. To overcome this problem, a method to inject donor SSCs into the rete testis of large animals

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under ultrasound guidance was developed. However, a previous study showed that the rete testis cannot be identified in stallions using sonography [24]. Therefore, instead of injection into the rete testis, 5 mL of iodixanol-contrasted fluorescein solution was injected into the parenchyma below the epididymis, which helped identify contrast in the vessels extending toward the head of the epididymis. Thus, this previous finding indicated that the testicular injection method may be applicable for SSCT in stallions. Hence, we transplanted SSCs directly into the testicular parenchyma of stallions without identifying the rete testis by sonography; however, we found no donor-derived sperm in the recipient semen after transplantation. This approache revealed an additional issue that the recipient pose and anesthesia should be considered for the successful transplantation. Because since the recipient horse was standing without anesthesia while in performing transplantation, even if the needles were properly inserted into the testicular parenchyma, the needle position and germ cell migration might be interrupted by continuous movement of the recipient. However, the general anesthesia in horses carries a mortality rate 1 in 10 due to they have heavy muscles and organs, which do not function while standing, as well as low blood oxygen levels, which can also cause problems. Moreover, the expertise in anesthesia, appropriate drugs, and sepcific equipments, along with a suitable environment, are required. Thus, the testicular injection of donor SSCs without considering the pose and anesthesia of the recipient is not an optimal transplantation techniques for producing donor-derived sperm [25]. Therefore, alternative approach must be considered to obtain donor sperm without transplanting germ cells into the testes of recipient stallions. Several techniques, such as in vitro single germ cell culture [72], testicular tissue culture [73, 74], xenograft [75], and xenotransplantation [76, 77], have been introduced to produce donor sperm without transplanting SSC in recipient testis. However, their efficacy in stallions has not yet been confirmed. Overall, the current experimental approaches used to produce donor SSC-derived sperm in stallions are inefficient, and clarity regarding the alternative methods is lacking. Additionally, factors such as enrichment of donor SSCs and preparation of germ cells with mitotically active Sertoli cells must be considered to ensure successful transplantation. Finally, alternative approaches to obtain donor sperm without transplanting germ cells into the testes of recipient stallions are required.

**Summary**

According to recent research in the field of animal breeding, alternative and innovative breeding techniques are being widely explored to efficiently breed animals with superior genetics. These techniques include artificial insemination (AI), somatic cell nuclear transfer technology (SCNT), and SSCT. A concept that is being actively

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221 investigated in this field of research is the utilization of feeder mermaphrodite-based SSC. The aim of this
222 approach is to breed and disseminate superior biological genes in males and females without relying on female
223 animals. This greatly accelerates livestock breeding and production. However, several key factors, including long-
224 term in vitro culture and large-scale proliferation of SSCs, development of ideal recipient models, and effective
225 cell migration and migration pathways, must be addressed. While SSCT techniques for use in large animals are
226 currently available, obtaining successful results remains challenging. Therefore, further studies are required to
227 establish a reliable, efficient, and productive SSCT protocol for large animals.

228 *NANOS2* gene knock-out studies have primarily been performed using experimental animals, such as mice, but
229 recently, successful cases have been reported in larger animals. The infertile males generated through this gene-
230 editing technology have the capacity to successfully undergo SSCT. Hence, developing technologies to discover
231 and manipulate various genes involved in male germline maintenance to develop the ideal recipient animals is
232 necessary. Furthermore, research regarding genetic modification of SSCs should be accelerated to overcome high
233 costs and improve the efficiency of pronuclear injection and embryo-based genome modification techniques,
234 including SCNT. We suggested that this review may provide guidelines for future studies of spermatogonial stem
235 cell transplantation in the large animals that have not yet been performed.

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238 **Author Contributions**

239 Conceptualization, supervision, review, and editing: M.Y.; writing original draft, review, and editing:
240 H.J.. All authors have read and agreed to the final version of the manuscript.

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242 **Conflicts of Interest**

243 The authors declare no conflict of interest.

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